Development and validation of a HPLC-FLD method for the quantification of glyphosate and AMPA in drinking water

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Abstract

Glyphosate is a post-emergence organophosphate herbicide that eliminates and controls the spread of infesting plants. It is considered a non-toxic and non-carcinogenic herbicide, although several scientific studies assure the opposite. Due to this situation, the European scientific community has asked for a re-evaluation of glyphosate in which the results will be known by the end of the year 2022.

The main objective of this work was the development, optimization, and validation of an analytical methodology capable of quantifying glyphosate and AMPA in drinking water.

The analytical methodology used was High Performance Liquid Chromatography with Fluorescence Detection - HPLC-FLD, using the derivatization with FMOC-Cl that gives a chromophore to the molecule.

The validated methods allowed a quantification of glyphosate and its metabolite from 1 µg/L in a sample of drinking water, having obtained recoveries between 86 % and 104 % for the studied linearity range of 1 - 20 µg/L.

Keywords: Drinking water, Glyphosate, AMPA, HPLC - FLD, FMOC-Cl, Derivatization

1. Introduction

Glyphosate is the most active herbicide among more than 750 different herbicides, with application in agriculture, forestry, urban and domestic uses. It is a non-selective systemic herbicide, widely used to combat weeds [1]. Glyphosate, chemically known as 2-(phosphonomethylamino acetic acid) (Figure 1) is the herbicide most used worldwide [2], as a post-emergence organophosphorus herbicide, that is, applied on the leaves of the weed plant enabling the elimination and control of propagation. Together with its degradation product, phosphonic acid (aminomethyl) (AMPA) (Figure 2), are the most detected substances in natural waters in many developed countries [3]. In Portugal, glyphosate is one of the most used pesticides and it is up to the General Directorate of Food and Veterinary Sciences to control the pesticides and the most appropriate periods for their research by management entities.[4]

Currently, the high use of glyphosate herbicide has been surrounded by controversy due to its effects on human health and the environment.[5] Therefore, the need for its monitoring/determination, particularly in the water matrix since glyphosate is highly soluble in water and through lixiviation of soils can lead to contamination of water bodies [6,7]. The research of pesticides in water intended for

human consumption is currently regulated by Decree Law No. 152/2017 of December 7, which amended Decree Law No. 306/2007 of August 27, which establishes the quality regime of water intended for human consumption and transposes into national law Directive 98/83/EC of the Council of 3 November and Directive (EU) 2015/1787 of the Commission of 6 October.[8,9]

It is in the scope of this problem that the present work arose, in which tests were performed in the water matrices, to optimize and validate an analytical methodology capable of quantifying glyphosate in drinking water. When analyzing glyphosate, mainly in water matrices, it is important to also analyze its degradation product, because there are certain situations where it is not possible to observe glyphosate, but only AMPA.

The objective of this dissertation was to develop an analytical method for the determination of glyphosate and its degradation product, AMPA phosphonic acid (aminomethyl) in the water matrix.

To this end, a sufficiently sensitive analytical methodology was optimized (High Performance Liquid Chromatography with Fluorescence Detection - HPLC-FLD) [11] in order to ensure reliable detection and measurement of glyphosate and AMPA concentration values, the implementation and validation of this method was carried out in the Laboratory of Analysis of the Instituto Superior Técnico (LAIST). One of the main objectives of LAIST is to contribute to the improvement of water quality through monitoring, both at the level of mandatory legislative parameters and through the analysis of others that are not mandatory but have a high relevance to public health.

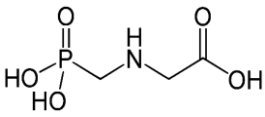

Figure 1- Glyphosate molecular structure

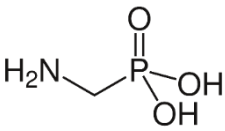

Figure 2- AMPA molecular structure

Glyphosate is a glycine analogous synthetic amino acid. It is stable in the air and does not degrade photochemically. Its degradation, made by some plants, leads to sarcosin and AMPA, the latter being the main product of degradation.[10] The AMPA, is a phytotoxin, and its occurrence with glyphosate can modify the physiology of the plant, to affect the photosynthesis, as well as induce oxidative stress [10]. Table 1 presents the physical-chemical properties of glyphosate and AMPA.

Table 1- Physico-chemical properties of glyphosate and AMPA.

Properties	**Glyphosate Characteristics**	**AMPA Characteristics**
Appearance	White powder	White powder
IUPAC Name	2-(phosphonomethylamino) acetic acid	(aminomethyl) phosphonic acid
Molecular formula	$C_3H_8NO_5P$	CH_6NO_3P
Molecular weight	169.07 g/mol	111,04 g/mol
Melting point	189.5 °C	189.5 °C
Solubility in water	10.5 g/L	-
Density	1.7 g/cm³	-

2. Experimental

2.1 *Chemicals and reagents*

Glyphosate (99.7%), AMPA (99.0%), FMOC-Cl (99.0%), Ammonium acetate (98 %), Potassium dihydrogen phosphate (99.5%), Hydrated sodium tetraborate (99.5%), Boric acid (99.8%), O-Phosphorus-L-Serine (99.8%), Acetonitrile (for HPLC- GOLD- Ultragradient); Dichloromethane (PESTIPUR for pesticide analysis), Methanol for analysis;

2.2 *Sample preparation*

A Borate buffer solution of 200 mM pH=8.98, a 5mM Ammonium Acetate buffer solution and a 50 mM Phthalate buffer solution were prepared. Once prepared, the solutions were placed for three minutes in an ultrasonic bath to degas.

As a derivatizer, an FMOC-CL 1.59 g/L solution was prepared, for this purpose about 15.9 mg of FMOC-CL were weighed and dissolved in acetonityl (ACN)and the resulting solution was taken to a final volume of 10 ml in a volumetric flask. The FMOC-CL solution was prepared again every 2 weeks and stored in the fridge at 4 ºC.

Glyphosate and AMPA mother standard solutions of 1 g/L contraction were prepared. From the mother solution, an intermediate solution of concentration 10 mg/L - Intermediate 1 was prepared. Another intermediate solution was prepared from the "intermediate 1" solution, with concentration equal to 1 mg/L - Intermediate 2. To draw the calibration curves, standard solutions were made based on the intermediate solutions prepared previously. The concentrations for the 5 standards of the calibration straight line range from 1 to 20 µg/L.

Two mobile phases were used: the first mobile phase consisted of mixing the phosphate buffer solution 50mM at pH 5.4 and acetonitrile with proportions (55:45) and the second mobile phase consisted also of mixing the ammonium acetate solution 5mM at pH 8.9 and methanol with proportions (80:20).

2.3 High Performance Liquid Chromatography

For the determination of glyphosate and its degradation product, AMPA phosphonic acid (aminomethyl) in the water matrix, the HPLC method was applied. HPLC is characterized using columns typically 15-25 cm long, with internal diameter of 2-5 mm, and filled with spherical porous particles with diameters of about 3-10 µm (stationary phase). The mobile phase crosses the column continuously at high pressures and with controlled flow and the mass transfer phenomena along the elution are favored by the increase of the contact area between the mobile and stationary phases, which translates into a decrease in height equivalent to a theoretical plate.[12]

Still in partition chromatography there is normal phase chromatography, in which the stationary phase is more polar in relation to the eluent used in the mobile phase, and reverse phase chromatography, the one used and already mentioned in this work, in which the stationary phase is less polar than the mobile phase. The most common stationary phases in the reverse phase are amino, phenyl, cyan, C4, C8, C18.

A HPLC equipment, consists of several components that allow the quantification and identification of compounds to be analyzed. The system is always coupled to a computer where the translation/reading of the analytes that were detected in the detector is done. [12]

2.4 Experimental procedure

2.4.1 Derivatization conditions

The samples were taken from the cold until they reached room temperature. In bottles suitable for the procedure, first 1 mL of the sample was added and then 0.2 mL of borate buffer was added. After waiting 10 minutes, 0.2 mL of water was added and then 50 µL of acetonitrile and 150 µL of FMOC-Cl solution were added. The samples rested for about 60 minutes.

Then a washing process was carried out where 1mL of dichloromethane (DCM) was added to the samples, the vortex was shaken for 1 min and the phases were separated. DCM was carefully removed, and the procedure was repeated once more.

2.4.2 HPLC operating conditions

For the present work, we used the operative conditions listed in tables 2 and 3 for column NH2 APS-2 HYPERSIL and column C8 Zorbax RX, respectively.

Table 2- HPLC operative conditions for Glyphosate and AMPA analysis in NH2 column APS-2 HYPERSIL.

Column temperature (ºC)	25ºC	
Injection volume (µl)	30	
Mobile Phase Flow Rate(mL/min); Isocratic Method	1	
Mobile Phase	A - 50 mM phosphate buffer 55%; B - acetonitrile 45%	
Time (min)	10	
Detector - FLD	PMT	15
	λ Excitation (nm)	265
	λEmission (nm)	315

Table 3- HPLC operative conditions for analysis of Glyphosate and AMPA in column C8 Zorbax RX.

Column temperature (ºC)	25ºC		
Injection volume (µl)	30		
Mobile Phase Flow Rate (mL/min)	1		
Gradient	t (min)	% A	% B
	0	80	20
	3	80	20
	6	30	70
	18	30	70
	23	80	20
	26	80	20
Mobile Phase	A - 5 mM Ammonium acetate; B - methanol		
Detector - FLD	PMT	15	
	λ Excitation (nm)	265	
	λEmission (nm)	315	

3. Results and Discussion

3.1 Tested methodologies

The objective of this work was to develop a chromatographic method for the determination of glyphosate and its degradation product, phosphonic acid (aminomethyl) in the water matrix. After consulting the literature and given the characteristics of the compound, in particular its polarity and therefore solubility in water, it was decided to select reverse phase partition liquid chromatography as the most appropriate methodology to be optimized. The chemical characteristics of glyphosate with respect to the absence of a chromophore group in the molecule impose some problems in its detection, being necessary to resort to a derivatization reaction in order to develop a sufficiently sensitive method with detection by molecular fluorescence for its determination and respective metabolite in water.

During the implementation of the methodology, the conditions of the derivatization process were optimized and the chromatographic process was varied, the choice of the column, the composition of the eluent as well as its pH, the injection volume and the cleaning process, the FLD detection conditions with respect to the excitation and emission wavelengths, and the use of the internal standard.

With respect to the derivatization process, it is important to emphasize that in the experimental procedure, the most well-known methodology is used, which is the reaction with FMOC-Cl, because besides conferring a chromophore to the molecule, it allows the use of a reverse phase chromatography method. Additionally, it also has the advantage of dispensing with the use of chlorinated organic solvents.

Two methodologies resulted from the set of tests performed, having in common the experimental procedure of derivatization:

- Methodology A in which once the derivatization process was finished the samples were read in the NH2 column APS-2 HYPERSIL using as mobile phase phosphate buffer and acetonitrile (55% Phosphate Buffer Solution + 45% ACN) and isocratic elution.
- Methodology B in which once the derivatization process was finished the samples were read in the C8 Zorbax RX column using as mobile phase ammonium acetate and methanol (80% ammonium acetate solution + 20% MeOH) and elution with gradient.

3.1.1 Methodology A

To test methodology A, the preparation of 200 µg/L concentration standards for glyphosate and AMPA was initially used to observe the compounds and their retention times. A typical chromatogram using the NH2 column APS-2 HYPERSIL and as eluent the mixture 55% Phosphate Buffer Solution + 45% ACN is shown in figure 3. As it can be verified the retention time of glyphosate is 6,185 minutes and its metabolite 2,751 minutes.

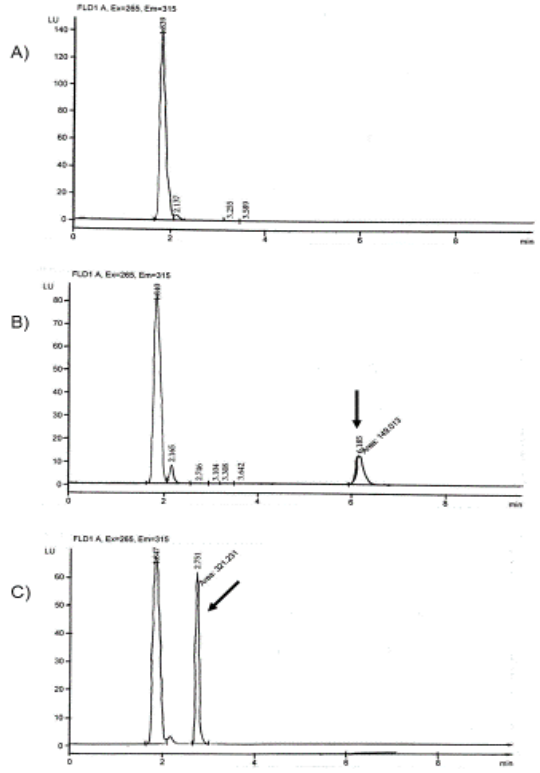

Figure 3- Chromatograms referring to methodology A tested from concentration standards of 200 µg/L, where A) White; B) Glyphosate with retention time equal to 6,185 min; C) AMPA with retention time equal to 2,751 min;

3.1.2 Methodology B

Methodology B basically consisted of changing the amine chromatographic column (NH2 APS-2 HYPERSIL) to the C8 column (C8 Zorbax RX). The derivatization procedure used was the same as Method A, however it was decided to change the glass bottles previously used for polipropylene test tubes to obtain better experimental results regarding the signal.

A typical chromatogram using the C8 Zorbax RX column is shown in figure 4, and as eluent

the mixture 80% Ammonium Acetate Buffer Solution + 20% MeOH with gradient elution. As can be seen the retention time of glyphosate is 2,600 minutes and its metabolite 7,007 minutes.

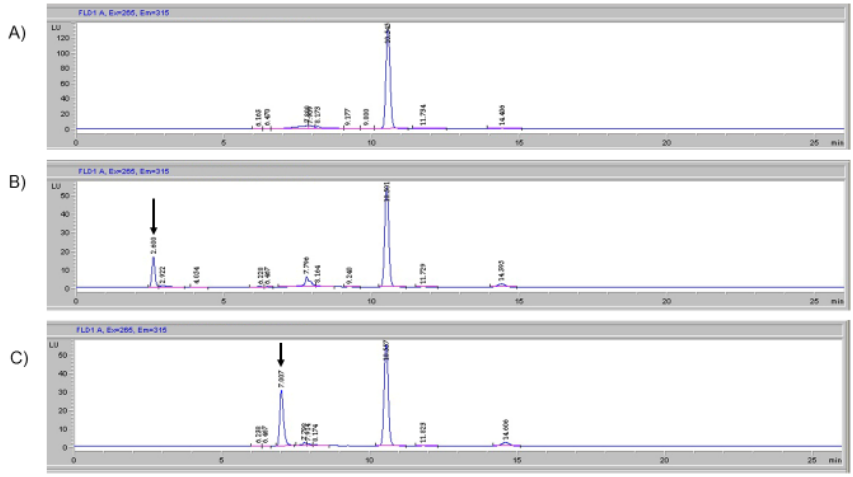

Figure 4- Chromatograms referring to methodology B tested from concentration standards of 200 µg/L, where A) White; B) Glyphosate with retention time equal to 2,600 min; C) AMPA with retention time equal to 7,007 min;

Based on methodology B, samples of tap water, underground water and superfecial water were recovered. In the first tests on tap water, 45% glyphosate and 64% AMPA were recovered and in relation to groundwater I, 96% glyphosate and 99% AMPA were recovered.

New tests were repeated on tap water, but this time sodium thiosulphate water was added to remove chlorine action and an average recovery for glyphosate of 93% was achieved.

For the new groundwater sample II, the recoveries are also reproducible in the range of 104% for glyphosate and 98% for AMPA.

In the case of surface water, the recovery values were not plausible, it is considered that these results may derive from the filtration process, which might not be the most appropriate for this type of sample containing certain contaminants that made it difficult to quatify the analytes in HPLC. [10,13]

3.1.3 Method validation

The method of external calibration was applied for quantification of glyphosate and AMPA. Calibration were constructed from 1 to 20 (the standard concentration levels to glyphosate and AMPA were at 1, 2.5, 5, 10 and 20 µg/L for methodology A and 1, 2.5, 5, 7.5, 15, 20 µg/L for methodology B). Calibration graphs were linear with good correlation coefficients (R^2) all being greater than 0,995.

Regarding the linearity of the methodologies, the linear responses were evaluated in terms of the tests (Mandel test, Rikilt test and residue analysis). The adjustments made allowed linearity for both methodologies.

The detection and quantification limits were determined for methodologies A and B, and LODs values were obtained in methodology A (glyphosate - 0.39 µg/L and AMPA - 0.52 µg/L) and for methodology B (0.47µg/L for glyphosate

and AMPA). Regarding the LOQs values, in methodology A it was obtained (glyphosate - 1.17µg/L and AMPA - 1.58 µg/L) and for methodology B (1.42 µg/L for glyphosate and AMPA).

The accuracy was calculated by relative standard deviations within the linear ranges. For groundwater I RSDs for glyphosate was 6% and for AMPA 2%, groundwater II was obtained RSDs for glyphosate 13% and AMPA 10%. Tap water samples (I) and (II) were also analyzed obtaining recoveries for glyphosate of 4% and 13%. The detailed accuracy data for each methodology and analites were shown in table 4.

Table 4- Results obtained for the study of accuracy in the drinking water matrices for methodology B

Methodology B	Groundwater I		Tap water (1)		Groundwater II		Tap water (2)
	GLY	AMPA	GLY	AMPA	GLY	AMPA	GLY
n	2	2	2	2	3	3	3
Average Rec (%)	96	99	45	64	104	98	93
Standard deviation	2	6	2	18	14	10	12
% RSD	2	6	4	28	13	10	13

3.2 Comparison between methodologies A and B

Table 5 compares methodologies A and B in terms of retention times, LOD, LOQ, theoretical plates numbers, H and the recovery percentages in the various types of water tested.

It should be noted that the retention times observed for glyphosate and AMPA in the two methodologies are different. This is due to the different stationary (columns) and mobile phases used in each methodology and to the different affinity of the analytes for each of the phases involved.

Based on the results of table 5, it can be concluded that methodology A is more sensitive than methodology B, this is due to the fact that the column used in this process (column NH2), presents a higher number of theoretical plates in relation to column C8 used in methodology B , that is, the two columns, no matter how big they are, the fact that they have different sizes in the particle, it is possible to distinguish between them which will have better sensitivity.

Regarding the recovery tests, these were tested from methodology B, because the column used in methodology A ended up deteriorating along the tests. However, acceptable recoveries were obtained for the water under study with only one particularity in surface water, which did not obtain plausible recovery results, namely due to the existence of interferences in the water that resulted in the difficulty of quantifying the analytes.

In short, due to the reasons explained above, methodology A proved to be the most suitable for the quantification of glyphosate and AMPA.

Table 5- Comparison of results obtained by methodologies A and B

		Methodology A		Methodology B	
		Glyphosate	AMPA	Glyphosate	AMPA
Retention time (min.)		6,185	2,751	2,600	7,007
Number of theoretical plates		(3,4±0,9) x10^3	(3,9±0,6) x10^3	(3,1±0,1) x10^3	(3,8±0,6) x10^3
H (cm)		4,34x10^{-3}	3,81x10^{-3}	4,77x10^{-3}	4,00x10^{-3}
LOD		0,39	0,52	0,47	0,47
LOQ		1,17	1,58	1,42	1,42
% Recovery	Tap water I			45	64
	Tap water II			93	86
	Groundwater I			96	99
	Groundwater II			104	98
	Surface water			-	-

4. Conclusions and Future Perspectives

The work performed consisted in the development, optimization and validation of an analytical method for quantification of glyphosate and AMPA in drinking water, based on the use of HPLC-FLD combined with an initial derivatization to make the target analyte detectable in the fluorescence spectrum.

The validation of the analytical method considered the need to meet the minimum requirements imposed by Decree Law No. 152/2017 of December 7. The limit of quantification of the HPLC-FLD method for the analysis of glyphosate and its metabolite was 1 µg/L, being a value higher than the limit imposed by law of 0.1 µg/L.

Regarding the method validation, the linearity was a parameter to be studied and as working range the range between 1-20 µg/L was chosen. This linearity range was studied fulfilling the requirements of the statistical tests studied, such as the Mandel test, Rikilt test and residue analysis. The accuracy was also studied and standard deviations between 2-18 % were obtained.

In the validation of the global method for the quantification of glyphosate and AMPA in drinking water, two methodologies A and B were optimized, in which derivatization using as FMOC-Cl reagent was ensured.

Between the two methodologies studied, methodology A using column NH_2 was the most appropriate for this method, as lower concentrations of 0.1 µg/L were achieved, but the fact that the column deteriorated after some tests made it impossible to treat more tests in it and thus the studies in column C8 continued.

With the confinement generated by the pandemic - COVID-19, which is currently being lived, it was not possible to perform more tests in favor of new knowledge because the difficulty in finding material for new experiments was greater.

Since the laboratory provides the UPLC apparatus for internal studies, it would be good to continue to develop more tests related to glyphosate in order to compare with the tests done previously on HPLC, but due to the current situation, it was not possible.

It is necessary to stress that the use of FMOC-Cl in the process of derivatization has some problems in the lifetime of the chromatographic column detecting it more easily. Therefore, I think it would be beneficial to develop a new technique of centrifugal nanofiltration, which consists of obtaining concentration factors higher than 20, reducing the volume of samples to 100 µL of concentrate.

Since, this technique could be used to replace the use of the FMOC-Cl and provide a longer time of use of the column in relation to the tests of the studied analytes.

In this case, for few more tests performed to detect and optimize glyphosate and AMPA, based on this project it was possible to acquire new knowledge of liquid chromatography even without experience in this area.

However, it was an enriching and valuable knowledge for my future, and it gave me great pleasure to work even with all the warnings on the route.

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